The When, What & How of Measuring Vitamin D Metabolism

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Part I

General Introduction

Chapter 1

The When, What & How of Measuring Vitamin D Metabolism in Clinical Medicine

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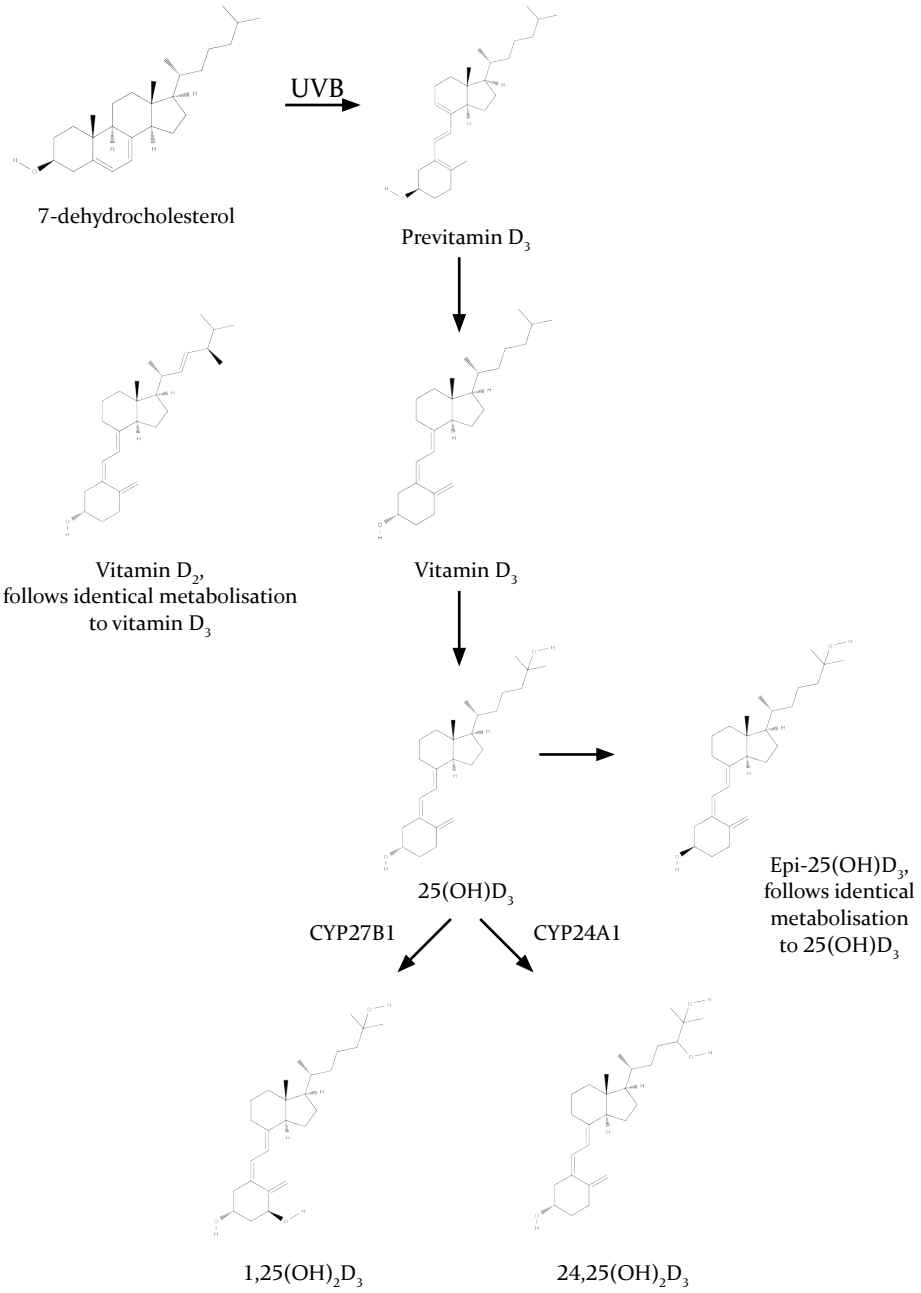

Figure 1. Structures of the different vitamin D metabolites discussed in this thesis. 25(OH)D: 25-hydroxyvitamin D; $1,25(OH)_2D$: 1,25-dihydroxyvitamin D; $24,25(OH)_2D$: 24,25-dihydroxyvitamin D

VITAMIN D METABOLISM

Vitamin D metabolism (Figures 1 & 2) is a complex and ingenious process proceeding inside multiple organs within the human body to maintain calcium homeostasis and control bone metabolism. It all starts with 7-dehydrocholesterol which, when exposed to UVB light in the human skin, transforms into vitamin D_3 through intermediate isomer pre-vitamin D_3. Alternatively, a small portion of vitamin D_3 may be directly ingested by the consumption of animal products. Another isomeric form, vitamin D_2, is not synthesised by our own body, but may be extracted from some plants and fungi. Both forms behave similarly and undergo the same metabolic process. Both activated forms also bind to the vitamin D receptor (VDR), although the active metabolite of vitamin D_3 has proven to be more potent [1]. From here, if there is no need for the distinction between the two isomers, vitamin D and associated metabolic products refer to both species. Cutaneously synthesised or ingested vitamin D is subsequently transported to the liver, usually bound to a vitamin D binding protein (DBP), where hydroxylation on the 25-position by hepatic CYP2R1 (25-hydroxylase) yields 25-hydroxyvitamin D (25(OH)D). The hormonally active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is mainly produced in the proximal renal tubule by the mitochondrial CYP27B1 enzyme (1α-hydroxylase), after a second hydroxylation at the 1-position. To retain $1,25(OH)_2D$ concentrations within the strict boundaries required for appropriate calcium homeostasis and bone metabolism, both $1,25(OH)_2D$ and 25(OH)D may undergo further hydroxylation by renal CYP24A1 (24-hydroxylase), leading, respectively, to 1,24,25-trihydroxyvitamin D and 24(R),25-dihydroxyvitamin D ($24,25(OH)_2D$), both having negligible affinity for the VDR. In addition, all of the aforementioned metabolites are subject to C-3 epimerisation resulting in products with lower VDR and DBP binding affinities and reduced activity compared to their unepimerised counterparts [2]

This metabolic process is tightly controlled by a number of regulators. Parathyroid hormone (PTH))—secreted by the parathyroid glands upon decreased calcium concentrations—stimulates production of $1,25(OH)_2D$ by upregulating CYP27B1 gene expression, while inhibiting expression of CYP24A1. Fibroblast

growth factor 23 (FGF23)—secreted by osteocytes in response to increased 1,25(OH)2D concentrations—inhibits CYP27B1 and stimulates CYP24A1, thus preventing the production of $1,25(OH)_2D$ and stimulating its catabolism [3].

Calcium decreases CYP27B1 activity directly and through inhibition of the action as exerted by PTH, both leading to lower $1,25(OH)_2D$ concentrations. Lack of phosphate (hypophosphatemia) results in the opposite, by stimulating CYP27B1 and inhibiting CYP24A1 expression. $1,25(OH)_2D$ itself suppresses CYP27B1 and stimulates CYP24A1, thereby promoting its own degradation when excessively present. Apart from the well-documented renal conversion of 25(OH)D to $1,25(OH)_2D$ for endocrine purposes under the regulatory control of PTH, FGF23 and $1,25(OH)_2D$, other vitamin D activating sites exist. In fact, many tissues harbour cells capable of metabolising 25(OH)D and additionally express the VDR, resulting in the possibility of intracrine, autocrine and paracrine effects [4]. These include immune cells, different types of epithelial cells, bone cells and parathyroid cells. Control of $1,25(OH)_2D$ production in these extrarenal cells differs from regulation within the kidneys, as receptors for PTH are lacking. Here, the most important limiting factor is the availability of substrate [5]. The non-classical actions of $1,25(OH)_2D$ in these cells involve local regulation of PTH secretion in the parathyroid gland, local regulation of insulin secretion in the pancreatic beta cells, modulating immune cell response in activated inflammatory

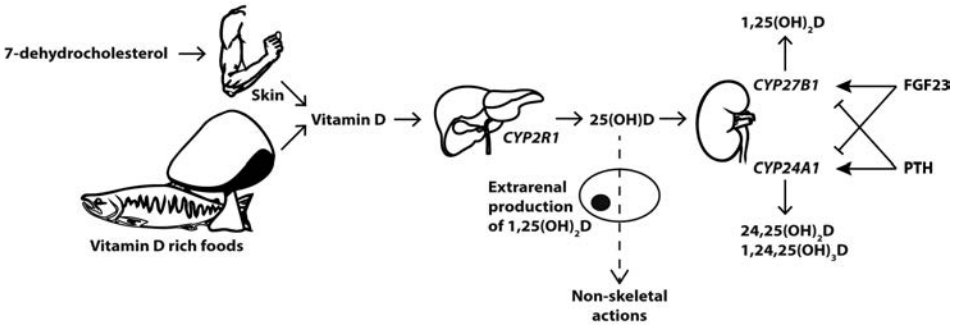

Figure 2. Vitamin D metabolism. From the production of vitamin D in the human skin or ingestion from vitamin D-rich foods to the final metabolisation into active (1,25(OH)2D) and largely inactive metabolites (24,25(OH)2D and 1,24,25(OH)3D) under regulation of PTH and FGF23. 25(OH)D: 25-hydroxyvitamin D; 1,25(OH)2D: 1,25-dihydroxyvitamin D; 24,25(OH)2D: 24,25-dihydroxyvitamin D; 1,24,25(OH)3D: 1,24,25-trihydroxyvitamin D; FGF23: fibroblast growth factor 23; PTH: parathyroid hormone.

cells and regulation of proliferation and differentiation in the keratinocytes [4]. Regulation of 25(OH)D metabolism, mediated by the two cytochrome P450 enzymes, is of crucial importance. When out of balance, serious complications may occur. Too high $1,25(OH)_2D$ concentrations result in increased calcium absorption from the intestines leading to hypercalcaemia and associated serious effects. Too low concentrations will mean a shortage of free calcium or hypocalcaemia and the accompanying symptoms. As such, $1,25(OH)_2D$, is almost perpetually within normal limits, even when, based on 25(OH)D concentrations, the patient displays vitamin D deficiency.

WHEN & WHAT: ASSESSING VITAMIN D METABOLISM MISBALANCE

25(OH)D

Serum total 25(OH)D, the summation of $25(OH)D_2$ and $25(OH)D_3$, is the best reflection of vitamin D status. It is a better marker for vitamin D status than circulating $1,25(OH)_2D$, as the latter is tightly regulated and its levels are strictly kept between limits even when adverse effects start to occur. Next to renal 25(OH)D metabolism, the local extrarenal conversion of 25(OH)D into $1,25(OH)_2D$, accounts for the hormone's non-calcaemic effects. Notably, these extrarenally produced $1,25(OH)_2D$ levels will not be mirrored by the concentration of $1,25(OH)_2D$ in the systemic circulation if the locally produced $1,25(OH)_2D$ does not leave the extracellular confinements to enter the bloodstream. In contrast, circulating 25(OH) D concentrations reflect the cellular 25(OH)D concentrations prior to conversion and as such, represent the available substrate for extrarenal 1,25(OH)D synthesis. Advantageously, as 25(OH)D is the main circulating metabolite of vitamin D, high concentration facilitates its measurement.

Experts in the field of vitamin D have provided us with several reviews on the measurement of vitamin D status and interpretation of the results [6-9]. Nevertheless, disagreement still prevails, as consensus on the precise 25(OH) D concentrations representing vitamin D deficiency, insufficiency, sufficiency and intoxication has not yet been reached. One could argue that the 25(OH)D concentrations found in representative groups of individuals, asymptomatic for

disease, are to be considered normal and desired [10-12]. However, many experts believe, as a result of changing diets and reduced levels of sun exposure, a large part of the world population is not vitamin D sufficient, and should therefore be supplemented [7-9, 13, 14]. They are convinced these higher 25(OH)D concentrations are necessary to prevent morbidity. According to the Institute of Medicine, serum 25(OH)D levels of 50 nmol/L (20 ng/mL) are sufficient to ensure skeletal health and only levels below 30 nmol/L (12 ng/mL) are to be considered universally inadequate, while levels between 30 and 50 nmol/L (12–20 ng/mL) potentially are, depending on the individual [15]. The Endocrine Society defines deficiency as 25(OH)D levels below 50 nmol/L (20 ng/mL) but recommends at least levels above 75 nmol/L (30 ng/mL), and preferably between 100 and 150 nmol/L (40–60 ng/mL), achieved by daily supplementation. However, both institutes deem population wide screening for vitamin D deficiency unnecessary and advise only to screen populations at risk [16]. Such groups include obese individuals, pregnant and lactating women, individuals with darker skin pigmentation, older adults with a history of falls or fractures, patients with rickets, osteomalacia, osteoporosis, chronic kidney disease (CKD), liver failure, hyperparathyroidism, granuloma-forming disorders, some lymphomas, malabsorption issues or nephrotic syndrome and patients on a wide range of medications, including anticonvulsants, glucocorticoids, antifungals and medication to treat HIV/AIDS [15, 16].

$1,25(OH)_2D$

Counterintuitively, measuring $1,25(OH)_2D$ as a marker for vitamin D homeostasis is irrelevant in most cases, as it will be within reference range, even when one is considered vitamin D deficient and experiences associated adverse effects. Specific conditions, however, may require the assessment of $1,25(OH)_2D$, of which concentrations normally range between 59 and 159 pmol/L (25–66 pg/mL) [17]. Several diseases may either increase or decrease $1,25(OH)_2D$ concentration to undesirable levels [6]. These are all characterised by disturbed vitamin D metabolism on the level of $1,25(OH)_2D$ production, which is often not reflected by altered 25(OH)D concentrations. The disorders may be classified according to three distinct origins of the $1,25(OH)_2D$ production imbalance. First, and most

common are disorders of the $1,25(OH)_2D$ producing CYP27B1 enzyme. A second arises from mutations in the vitamin D receptor (VDR), rendering it unresponsive or less responsive to its substrate. Finally, a third category consists of conditions characterised by excessive extrarenal conversion of 25(OH)D into $1,25(OH)_2D$. 1α-hydroxylase deficiency, also known as vitamin D-dependent rickets type 1 or pseudo-vitamin D deficiency rickets, is an autosomal recessive disorder exemplary for the first category. This rare disease is caused by an inactivating mutation in CYP27B1, resulting in abnormally low $1,25(OH)_2D$ concentrations and early onset of rickets [18]. A number of disorders presenting as hypophosphatemic syndromes also belong to the first category. Many, but not all, of these syndromes are mediated by FGF23, and $1,25(OH)_2D$ is an excellent marker to differentiate between the FGF23-mediated and non-FGF23-mediated syndromes. The genetic disorders X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), and autosomal recessive hypophosphatemic rickets 1,2 and 3 (ARHR1, ARHR2 and ARHR3), are all examples of the FGF23-mediated kind, as is the acquired tumour induced osteomalacia (TIO) [19-26]. Additionally, other rare disorders may manifest as FGF23-mediated hypophosphatemia, including osteoglophonic dysplasia, McCune–Albright syndrome, epidermal nevus syndrome, neurofibromatosis, hypophosphatemic rickets with hyperparathyroidism and Jansen metaphyseal chondrodysplasia [27-32]. Elevated FGF23 levels result in inhibition of CYP27B1 and subsequent low or inappropriately normal $1,25(OH)_2D$ concentrations [33]. The non-FGF23-mediated disorders displaying hypophosphatemia, including hereditary hypophosphatemic rickets with hypercalciuria, idiopathic hypercalciuria and Fanconi syndrome display similar phenotypes yet, in contrast to the FGF23-mediated syndromes, result in normal FGF23 levels and normal or appropriately elevated $1,25(OH)_2D$ concentrations [34-36]. Hereditary vitamin D-resistant rickets, also known as vitamin D-dependent rickets type 2 belongs to the second category and is caused by a mutation in VDR, rendering it unresponsive to its substrate, resulting in hypocalcaemia and early onset rickets. Very high $1,25(OH)_2D$ concentrations are found in these individuals. Examples of the third category, sarcoidosis, tuberculosis, rheumatoid arthritis, inflammatory bowel disease and the lymphoproliferative disorders, are all characterised by the formation of lumps

of inflammatory cells, or granulomas, with the capability of hydroxylation of 25(OH)D to form 1,25$(OH)_2$D, facilitating antimicrobial and anti-inflammatory immune responses. This extrarenal 1α-hydroxylation by local CYP27B1 is not controlled by PTH, FGFG23, phosphate or 1,25$(OH)_2$D, but is regulated by local factors such as IFN-γ and IL15 and dependent on the availability of substrate [37]. When excessive, the locally produced 1,25$(OH)_2$D may escape the confinements of the intracellular space, spill over to the systemic circulation, and raise blood 1,25$(OH)_2$D concentrations to abnormally high levels [38]. Importantly, extrarenal 1-hydroxylation is often associated with low bone mineral density. 1,25$(OH)_2$D assessment is therefore not only of great clinical value in the diagnosis of the disease but also in the prevention of complications in the form of bone disorders [39, 40].

In summary, requesting measurement of 1,25$(OH)_2$D could be of use on suspicion of 1α-hydroxylase deficiency where very low 1,25$(OH)_2$D concentrations are found. 1,25$(OH)_2$D measurement also aids in separating the disorders presenting as hypophosphatemic syndromes mediated by FGF23, where normal to low 1,25$(OH)_2$D concentrations are found, from the non-FGF23-mediated disorders with normal to high 1,25$(OH)_2$D concentrations. Very high 1,25$(OH)_2$D concentrations indicate hereditary vitamin D-resistant rickets or the presence of excessive extrarenal 1α-hydroxylation by granulomatous or lymphoproliferative diseases.

24,25$(OH)_2$D

The CYP24A1 enzyme catabolises excess 25(OH)D by converting it to the inactive 24,25$(OH)_2$D. Its expression is not limited to the kidneys. Similar to CYP27B1, it expands to many different cell types, allowing for the local regulation of 25(OH)D and 1,25$(OH)_2$D in target cells [41]. In a group of 92 healthy individuals evenly covering all age decades between 20 and 70 years, the 95% confidence interval of measured 24,25$(OH)_2$D concentrations ranged from 0.5 to 11 nmol/L (0.2–4.6 ng/mL) and the 25(OH)D/24,25$(OH)_2$D ratio ranged from eight to 27. Concentrations of 24,25$(OH)_2$D appeared to be independent from the presence or absence of vitamin D deficiency [42].

Two articles by St Arnaud in the late nineties elaborately postulated 24,25$(OH)_2$D as an additional active metabolite of 25(OH)D. A role in cartilage

development, embryogenesis and in regulating bone growth, development and repair was proposed [43, 44]. How 24,25$(OH)_2$D exerted these effects remained unclear, which led the authors to theorise on the existence of a specific nuclear or membrane receptor for 24,25$(OH)_2$D. To this day, researchers are still questioning the active physiological role of 24,25$(OH)_2$D and the possible existence of a dedicated receptor. Nonetheless, irrespective of its supposed activity, measuring 24,25$(OH)_2$D undeniably has value, as several studies have proven during the years following St Arnaud's articles. Unsurprisingly, the 25(OH)D/24,25$(OH)_2$D ratio is an indicator of CYP24A1 activity and vitamin D catabolism therewith [45, 46]. In that capacity, the ratio can identify a rare genetic disorder, idiopathic infantile hypercalcaemia (IIH). Infants suffering from this disorder display severe hypercalcaemia and suppressed PTH levels due to their severely impaired capacity to catabolise 25(OH)D and 1,25$(OH)_2$D, as a result of an inactivating mutation in the gene coding for CYP24A1 [46, 47]. Similarly, less harsh inactivating mutations may result in nephrocalcinosis and nephrolithiasis in adult life, secondary to hypercalciuria and often hypercalcaemia [48-50]. The effectiveness of the 25(OH)D/24,25$(OH)_2$D ratio was further established in a cohort of hypercalcaemic patients, in which it correctly identified those patients harbouring an inactivating CYP24A1 mutation [51]. Aditionally, an elevated 25(OH)D/24,25$(OH)_2$D ratio predicts vitamin D defiency to at least a similar extent as increased PTH does, and might be of potential use as an indicator of vitamin D deficiency [46, 52].

In conclusion, the 25(OH)D/24,25$(OH)_2$D ratio is an unambiguous marker for vitamin D catabolism and may identify patients with hypercalcaemia secondary to CYP24A1 mutations, such as patients with IIH.

OTHER METABOLITES

Beyond 25(OH)D, 1,25$(OH)_2$D and 24,25$(OH)_2$D many more vitamin D metabolites have been identified, yet these are scarcely measured. With the current state of techniques, many of the metabolites circulating at concentrations below that of 1,25$(OH)_2$D will be unquantifiable and the value of these metabolites for diagnostics remains elusive. Nevertheless, future studies may find ways of detecting and using these metabolites to provide new diagnostic tools in the years to come.

REGULATORS OF VITAMIN D METABOLISM

PTH is regularly measured as it is of great diagnostic value in hypo- and hypercalceamia and in patients with chronic renal failure, to diagnose secondary hyperparathryroidism.

Although FGF23 is not often requested from the clinic, in specific cases it can be of great diagnostic value. In the previous section on 1,25(OH)2D a number of conditions were described for which 1,25(OH)2D can discriminate between the FGF23-mediated and non-FGF23-mediated kind. Measuring FGF23 in the suspected FGF23-mediated hypophosphatemic syndromes is of course very helpful [53]. Circulating intact FGF23 protein is considered as the active hormone, but inactive C- and N-terminal fragments also circulate in the human bloodstream.

HOW: IMPORTANCE OF THE CHOICE OF METHOD

25(OH)D

25(OH)D can be measured in many different ways, yet two techniques dominate: automated immunoassay and liquid chromatography coupled to tandem-mass spectrometry (LC-MS/MS). According to the Vitamin D External Quality Assessment Scheme (DEQAS), overseeing method comparability across 54 countries, in October 2017, ~76% of the 871 participating laboratories used an automated immunoassay to measure their 25(OH)D, while ~18% used LC-MS/MS, ~3% used a manual immunoassay and ~2% used HPLC. During recent years, several studies addressed the limitations of the automated, and less frequently the manual immunoassays, when measuring in patients [54-59]. While in healthy individuals, the assays correlate nicely with the standardised LC-MS/MS method, measuring 25(OH)D in patient groups has revealed huge deviations [54, 57, 58, 60]. This brings to light some of the major issues with the automated and manual immunoassays. First, the lack of sample preparation in the automated immunoassays allows for the emergence of severe interferences from miscellaneous origins. Differing concentrations of DBP, which are greatly affected in certain populations, such as in pregnant women, intensive care patients and patients with liver failure, may be a common problem affecting results [57, 60, 61]. Similarly, the automated immunoassays often have

incomprehensible difficulties measuring 25(OH)D in haemodialysis patients and osteoporotic patients [54, 57, 59]. Unlike with the automated immunoassays, sample preparation for the HPLC, manual immunoassays and LC-MS/MS methods is adaptable and can be optimised to reduce any adverse effects that varying DBP concentrations or other contributing factors may bring. A second origin of bias for both the automated and manual immunoassays is related to the specificity of the used antibodies. As mentioned before, 25(OH)D may be present in one of two forms, $25(OH)D_2$ or $25(OH)D_3$. Immunoassays are generally not able to distinguish the two, and report these together as total 25(OH)D. As the antibodies in the immunoassay do not bind $25(OH)D_2$ to a similar extent as they bind $25(OH)D_3$, this leads to the under- or over-estimation of total 25(OH)D [62-66]. Alarmingly, many automated immunoassay manufacturers report considerably different cross-reactivity percentages for $25(OH)D_2$ compared to the observed values by independent researchers [67, 68]. This is particularly problematic in countries were supplementing with vitamin D_2 is more common, for example the USA. On the contrary, LC-MS/MS has no problem measuring $25(OH)D_2$ alongside $25(OH)D_3$, as the mass difference of 12 Da is easily separated by mass spectrometry. Another inherent specificity problem for the assays using antibodies, is cross-reactivity with other vitamin D metabolites. $24,25(OH)_2D_3$, present in about 10–15% of the 25(OH)D concentration, has proven to greatly impact immunoassays and produces falsely high results [52, 69, 70]. Similar to $25(OH)D_2$, $24,25(OH)_2D_3$, has a different mass to $25(OH)D_3$ and poses no threat to the reliability of LC-MS/MS methods [69]. Epi-25(OH)D, the result of epimerisation of 25(OH)D, with levels that are highest in neonates up to one year of age and present in most adults at about 9% of 25(OH)D, does not influence the results of immunoassays, as antibodies do not recognise it [63, 71, 72]. Many LC-MS/MS assays, on the other hand, experience difficulties distinguishing the two epimers due to their equal mass and their similar affinity for most LC columns, resulting in an overestimation, and potential misclassification, of total 25(OH)D [63, 71, 73, 74]. Several LC-MS/MS assays now use non-C18 columns enabling them to chromatographically separate the epimer and measure it independently of 25(OH)D, despite the identical masses [46, 70, 75-78].

When the first LC-MS/MS methods for 25(OH)D made their entry, DEQAS

reported unflatteringly high interlaboratory differences in the measurement of 25(OH)D. Carter and Jones showed these differences were often attributable to the use of in-house calibrated standards, and significantly abated after the use of a shared standard [79]. Indeed, another study showed LC-MS/MS methods for 25(OH)D generally correlated nicely and small biases originated from differences in calibration procedures [80]. Thanks to the Vitamin D Standardization Program (VDSP) and the target value DEQAS now provides for participants, laboratories operating miscalibrated LC-MS/MS methods have the possibility to standardise their methods using the reference measurement procedure, which is crucial for their performance.

In earlier years, automated immunoassays had largely replaced manual assays with their easy operation and higher throughput. However, all the aforementioned flaws of the immunoassays that were brought to light in certain patient groups, have now tipped the scales in favour of the LC-MS/MS. Today, LC-MS/MS is slowly replacing automated immunoassays in clinical laboratories. Due to the necessity of more complex and expensive equipment and highly skilled technicians, most of the smaller laboratories still depend on the manual and automated immunoassays. Manual immunoassays may still be well-suited for 25(OH)D measurement in patient groups where adequate sample preparation resolves the specificity issues of the technique. Automated immunoassays may still be suitable for the determination of 25(OH)D concentrations in large cohorts of healthy individuals, where interferences and supplementation are less frequent. We must conclude however, that measuring 25(OH)D in a clinical setting benefits greatly from the transition from automated immunoassays to LC-MS/MS. Still, attention to the specifications and quality of LC-MS/MS methods is advised. Particular specifications, such as separately quantifying epi-25(OH)D_3 in neonates, may be crucial. Standardisation and the resulting good agreement with the DEQAS target value or performance within the VDSP defines high quality methods. Without data on method agreement, DEQAS performance or standardisation, the quality and suitability of a method is indefinable and the possibility of misclassification exists.

Of note, a few laboratories still run HPLC methods for their 25(OH)D determination. While most of the advantages of LC-MS/MS over immunoassays also hold true for HPLC, mass spectrometers have largely replaced the UV detectors

used in combination with HPLC, for their superior sensitivity and specificity. The advantages and disadvantages of today's two most widely used techniques, immunoassays and LC-MS/MS for 25(OH)D measurement are summarised in Table 1. All things considered, LC-MS/MS is the preferred technique for 25(OH)D measurement in patients, without the knowledge of any present interferences that might hinder its determination. The use of immunoassays is not advised in specific patient groups, including IC, haemodialysis, osteoporotic and liver failure patients, pregnant women, neonates and individuals on D_2 supplementation, and caution is warranted in unstudied patient groups.

Table 1. Advantages and disadvantages of current LC-MS/MS methods and immunoassays for vitamin D metabolism determination.

Metabolite	**LC-MS/MS**		**Immunoassay**	
	Advantages	*Disadvantages*	*Advantages*	*Disadvantages*
25(OH)D	-Sample preparation adaptable -Specificity	-Complexity -Difficult to separate epi-25(OH)D_3 from 25(OH)D_3	-Fast* -Easy operation* -No cross reactivty with epi-25(OH)D_3	-Patient group-dependent deviations -No distinction between 25(OH)D_2 and 25(OH)D_3 -Cross-reactivity of other vitamin D metabolites
	Advantages	*Disadvantages*	*Advantages*	*Disadvantages*
1,25(OH)$_2$D	-Sample preparation adaptable -Specificity	-Complexity -Sensitivity -Possible cross-reactivity from isobaric interferences	-Fast -Easy operation Sensitivity	-No distinction between 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$ -Cross-reactivity of other vitamin D metabolites recognised by the antibody
	Advantages	*Disadvantages*	*Advantages*	*Disadvantages*
24,25(OH)$_2$D	-Sample preparation adaptable -Specificity	-Complexity	N/A†	N/A†

*The advantages listed are limited to the automated immunoassays. †Although 24,25(OH)$_2$D immunoassays exist, too little information on their performance is available.

$1,25(OH)_2D$

As a result of the very low concentrations of circulating $1,25(OH)_2D$ and the specialised circumstances under which its analysis is deemed necessary, fewer laboratories conduct this measurement. Of the 165 methods that participated in the DEQAS distribution of October 2017, ~75% of laboratories used an automated immunoassay to measure $1,25(OH)_2D$, ~17% used a manual immunoassay and ~9% used LC-MS/MS. Similar to measuring 25(OH)D, the methods for measuring $1,25(OH)_2D$ each have their advantages and disadvantages (Table 1), making the selection of one of them difficult and relevant. Previously, the field of $1,25(OH)_2D$ measurement was dominated by radio-immunoassays. These assays often suffered from substantial cross-reactivity with other vitamin D metabolites and were unable to produce evincive results [81, 82]. In the case of the $1,25(OH)_2D$ assays, the newest automated immunoassays perform similarly to LC-MS/MS and no apparent specificity problem exists. Here, the overall unaccountable lack of good correlations between assays on all platforms is most problematic [17, 83, 84]. Without the presence of a reference method, identifying the methods that deviate is very difficult, if not impossible. A recently developed automated immunoassay shows good correlations to some of the LC-MS/MS methods that include an immunopurification step in their sample preparation, while lesser correlations are found with others that do not [84-86]. LC-MS/MS methods without immunopurification included in their sample preparation might suffer from isobaric interferences, such as 1β-25-dihydroxyvitamin D_3, which would not be co-captured by the antibodies in the automated immunoassay [87]. Additionally, as with 25(OH)D, differences in calibration procedures are a likely cause of deviations and methods would substantially benefit from a target value provided by DEQAS and subsequent standardisation.

While immunoassays are quite easily capable of measuring very low concentrations of $1,25(OH)_2D$ due to the inherent amplifying nature of the technique, LC-MS/MS assays struggle gaining enough sensitivity to accurately measure in the lower ranges of physiological concentrations. One way to overcome this is the use of more sophisticated chromatography. By making use of a 2D chromatography system,

background noise is reduced and befouling of the MS instrument diminished. It comprises of two parallel connected columns. Only when the analytes of interest elute from the first column is it connected to the second column. Before and after this moment, flow is directed towards a waste. Another option is derivatisation of the vitamin D molecule. By chemical transformation of the molecule into something more easily ionised, sensitivity is improved. For the vitamin D molecules, several derivatisation agents are available. Most of them are based on the same Diels–Alder reaction taking place on the diene structure adjacent to the A-ring in the backbone of vitamin D. The diophenile molecules differ in the groups attached to their triazole backbone. The two most widely used derivatisation agents are PTAD and Amplifex.

As expected, the assays using a derivatisation agent generally report lower limits of quantification (LOQs), with levels up to 2.5 pmol/L [17, 77, 82, 88-90]. The assays not using derivatisation require more elaborative sample preparation (immunopurification), increased sample volume (>0.5 mL), more sophisticated LC-MS/MS systems (μLC or 2D chromatography) or do not reach desirable LOQs [76, 90-94]. The lowest LOQs have been reached using immunopurification followed by PTAD derivatisation [17, 82]. Using Amplifex has the advantage of not requiring an immunopurification step, which makes it possible to include additional vitamin D metabolites aside from 1,25(OH) D_3 and 1,25(OH)D_2, but impedes the chromatographic separation of epimers.

It is difficult to convincingly argue in favour of one technique over the other in the case of 1,25$(OH)_2$D. When separate quantification of 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ is preferable, in countries with vitamin D_2 supplementation, LC-MS/MS is the only option. Notwithstanding, in the case of 25(OH)D, standardisation and subsequent amelioration of LC-MS/MS methods exposed the pitfalls of the immunoassays. With the 1,25$(OH)_2$D methods, a similar chain of insights may lay ahead. Without the availability of a reference method, though, standardisation is not an option and identifying the origin of biases between methods and prescribing necessities for 1,25$(OH)_2$D measurements in clinical diagnostics is difficult. Nonetheless, three things are evident from the available data. First, there is no incontestable difference of outcome between the newest automated immunoassays and LC-MS/MS. Second, when using a dedicated LC-MS/MS method

for $1,25(OH)_2D$, incorporation of immunopurification in the sample preparation generally leads to a method with greater sensitivity and specificity. Third, when measuring multiple vitamin D metabolites at the same time, immunopurification is not applicable and LC conditions and sensitivity optimisation need considerable attention to accurately measure each metabolite at endogenous concentrations.

$24,25(OH)_2D$

Several laboratories have developed methods for the quantification of $24,25(OH)_2D$. Those participating in the DEQAS distribution all use LC-MS/MS, despite the existence of a $24,25(OH)_2D$ radio-immunoassay. The advantages and disadvantages of LC-MS/MS methods are depicted in Table 1. Most of them, but not all, use an isotopically labelled standard to aid them in accurate measurement. The methods measuring $24,25(OH)_2D$ have usually incorporated additional vitamin D metabolites in their analysis. One thing to consider is the application of a derivatisation agent. $24,25(OH)_2D$ is present at about 10% of the 25(OH)D concentration. As a result, similar to $1,25(OH)_2D$, it is not easily measured by our current high-end LC-MS/MS systems without extra efforts to improve sensitivity. One of the options is derivatising the metabolite with a derivatisation agent, like PTAD, DMEQ-TAD or Amplifex [46, 77, 90, 95]. This increases sensitivity considerably, yet unfortunately PTAD thwarts the separate quantification of epi-$25(OH)D_3$. Without derivatisation, higher LOQs are reported, additional efforts in other areas have to be undertaken to acquire similar LOQs, or higher sample volumes are required [45, 70, 75, 76, 88, 96, 97].

A candidate reference method was published by Tai and Nelson with high precision and accuracy [98]. Using five serum samples identified by the NIST as Standard Reference Material (SRM) and 30 samples from the DEQAS distribution, the candidate reference method and five laboratories routinely measuring $24,25(OH)_2D$ assessed their comparability [99]. Mean biases of the participants ranged from –15% to 36% for the SRM samples and from 6% to 15% for the DEQAS samples. A significant SD of the mean bias for some of the laboratories indicated room for precision improvement. Overall, significant biases with the candidate reference method for $24,25(OH)_2D$ shows we are long from uniform measurement of the metabolite. This is especially problematic

when measuring the 25(OH)D/24,25$(OH)_2$D ratio as it becomes unreliable and inaccurate when either one of the two methods is unstandardised. Further efforts for standardisation, including the availability of the SRMs and provision of a target value by DEQAS, are a necessity for uniform measurement and interpretation.

The measurement of 24,25$(OH)_2$D remains mainly research driven instead of clinical, and data on method comparison is scarce. In consequence, designating high quality methods is difficult. In the coming years, 24,25$(OH)_2$D will probably be more widely incorporated in clinical laboratories and attention to standardisation will lead to its solid anchoring within vitamin D metabolism diagnostics.

OTHER VITAMIN D METABOLITES

A number of the aforementioned LC-MS/MS methods measure additional metabolites. Where, in the case of epi-25(OH)D3, the value of measurement in neonates is well established, for other metabolites, such as 4β,25(OH)2D3, 23(R),25(OH)2D3 and 24(OH)D, the significance is not clear. These metabolites are usually incorporated in LC-MS/MS methods after being identified as an interference, and subsequently chromatographically separated and quantified. To date, a real reason to quantitatively measure these additional vitamin D metabolites is lacking, and nothing can be stated about the importance of the choice of method.

REGULATORS OF VITAMIN D METABOLISM

Not all circulating PTH is composed of the active form (1-84). Many other C-terminal, N-terminal and middle fragments circulate in the bloodsteam. Second generation PTH assays measure the active intact protein (1-84) and some of the bigger fragments (mainly 7-84), while the third generation assay is specific only for the intact protein as it uses a antibody directed towards the first 4 amino acids [100]. When studying vitamin D related disorders, the third generation PTH assays do not seem to perform better than the second generation assays.

FGF23 is inactivated by intracellular cleavage into a C- and N-terminal fragment. Next to the active intact protein, these inactive fragments circulate in the bloodstream. Measurement is currently possible with several immunoassays. Some

of these assays measure intact FGF23, while others measure both the intact FGF23 and the C-terminal fragment. These C-terminal assays are said to be less sensitive to pre-analytical instability compared to the intact FGF23 immunoassays and are therefore the preferable choice for most clinicians and laboratory specialists [101].

CONCLUSION

While the reasons for measuring 25(OH)D have often been described, the value of measuring its metabolites, 1,25(OH)2D and especially 24,25(OH)2D, remains largely unappreciated. In short, indications where measurement of 1,25(OH)2D may contribute to diagnosis or aid in monitoring treatment include conditions where production of 1,25(OH)2D is heavily disturbed, resulting in either a shortage of 1,25(OH)2D, such as in 1α-hydroxylase deficiency, or an overabundance of 1,25(OH)2D, such as in hereditary vitamin D-resistant rickets, sarcoidosis, tuberculosis, rheumatoid arthritis, inflammatory bowel disease and lymphoproliferative diseases. 1,25(OH)2D also helps to identify the hypophosphatemic syndromes mediated by FGF23, including XLH, ADHR, ARHR and TIO. Measuring 24,25(OH)2D enables identification of CYP24A1 mutations leading to impaired catabolism of the hormone and associated hypercalcaemia, such as in IIH. Measuring FGFG23 is very usefull in the identification of the FGF23-mediated hypophoshatemic syndromes.

OUTLINE OF THIS DISSERTATION

The aims of this dissertation were 1) to improve the quality, knowledge and the interpretation of measuring vitamin D metabolism and 2) describe clinical and research applications of the improved methods.

The first aim (methodology) is adressed in **part II** of this dissertation, which focusses on the methodology of 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$ and FGF23 measurement. The current phase of quality improvement differs for the different analytes. As a result, the focus of the chapters depends on the specific needs for method improvement for each specific analyte. **Chapter 2** describes the current status of 25(OH)D measurement, and more specifically, standardization. Various

routine 25(OH)D LC-MS/MS methods used in the Netherlands, applying different calibration procedures, are compared and their agreement is presented. **Chapter 3** describes the validation of a state-of-the-art method for determination of 1,25$(OH)_2$D. As minimal standardization efforts have been reported for 1,25$(OH)_2$D, we have incorporated a method comparison with a different LC-MS/MS method. Human reference values are also included to improve interpretation of measurements using this method. Similarly, **Chapter 4** describes an LC-MS/MS method for combined measurement of 25(OH)D and 24,25$(OH)_2$D and reference values for 24,25$(OH)_2$D and the 25(OH)D/24,25$(OH)_2$D ratio. With regard to FGF23, discussion exists whether the intact or the C-terminal immunoassays should be preferred. **Chapter 5** therefore discusses the contemporary immunoassays to measure intact FGF23 and C-terminal FGF23 and focusses on the pre-analytical stability of FGF23 with these assays.

The second aim (application) is addressed in **part III** of this dissertation. In **Chapter 6**, we examined the prevalence of vitamin D deficiency by assessing 25(OH)D and 24,25$(OH)_2$D levels in children with acute lymphoblastic leukemia in relation to methothrexate induced oral mucositis. **Chapter 7** describes the effect of vitamin D supplementation on the metabolism of vitamin D and discusses the possible value of measuring the 25(OH)D/24,25$(OH)_2$D ratio in predicting the change in 25(OH)D after supplementation.

Finally, in Chapter 8, the findings of all studies presented in this dissertation are summarized and discussed and an outlook into the possible future of measuring vitamin D metabolism is given.

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